c-Myc Overexpression Causes Anaplasia in Medulloblastoma

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Abstract

Both anaplasia and increased c-myc gene expression have been shown to be negative prognostic indicators for survival in medulloblastoma patients. myc gene amplification has been identified in many large cell/anaplastic medulloblastomas, but no causative link between c-myc and anaplastic changes has been established. To address this, we stably overexpressed c-myc in two medulloblastoma cell lines, DAOY and U228, and examined the changes in growth characteristics. When analyzed in vitro, cell lines with increased levels of c-myc had higher rates of growth and apoptosis as well as significantly improved ability to form colonies in soft agar compared with control. When injected s.c. into nu/nu mice, flank xenograft tumors with high levels of c-myc in DAOY cell line background were 75% larger than those derived from control. Over-expression of c-myc was required for tumor formation by U228 cells. Most remarkably, the histopathology of the Myc tumors was severely anaplastic, with large areas of necrosis/apoptosis, increased nuclear size, and macronucleoli. Indices of proliferation and apoptosis were also significantly higher in Myc xenografts. Thus, c-myc seems to play a causal role in inducing anaplasia in medulloblastoma. Because anaplastic changes are often observed in recurrent medulloblastoma, we propose that c-myc dysregulation is involved in the progression of these malignant embryonal neoplasms. (Cancer Res 2006; 66(2): 673-81)

Introduction

Medulloblastoma is the most common malignant tumor of the central nervous system in childhood. Treatment has improved greatly over the past few decades due to innovations in neurosurgical technique, better delivery of radiation therapy, and the advent of chemotherapy [reviewed by Rood et al. (1)]. For a subset of patients, overall survival is relatively high, approaching 70% to 80% (2–5). However, for patients with metastatic disease, with unresectable tumors, or who are very young, prognosis is worse (5, 6). Even for survivors, there is a high incidence of post-therapy morbidities (7). Current clinical trials are designed, in part, to identify biological features that will allow future stratification of therapy. This may improve outcomes for high-risk patients and reduce side effects in patients with lower-risk disease.

Although the presence of metastatic disease continues to be a strong predictor of poor outcome (3, 6), several additional prognostic markers have been identified. One histopathologic feature, large cell/anaplastic (LC/A) change, has been associated with poor prognosis [refs. 8–11; reviewed by Eberhart and Burger (12)]. The LC/A phenotype is defined by the presence of large pleomorphic nuclei, numerous mitoses, and an increased apoptotic rate; it is found in 20% to 25% of medulloblastoma (10, 13). Moderate or severe anaplasia was associated with a statistically significant difference in clinical outcome in a comprehensive retrospective review of Pediatric Oncology Group tumor specimens (10). Recent European studies have noted a similar incidence of medulloblastomas with LC/A features and confirmed the association with poor survival (11, 13).

The molecular mechanisms underlying the anaplastic phenotype are poorly understood. Several groups have found amplification of either the c-myc or the N-myc gene locus to be associated with the LC/A phenotype (9, 14–16). However, myc gene amplification is not universally detected in tumors with LC/A features, calling into question the linkage between increased myc activity and anaplastic change (13, 16–18). Additionally, when analyzed at a single-cell level by in situ methods, c-myc or N-myc amplification is sometimes present in only a subset of cells in a given tumor, including cells that do not appear anaplastic (15, 16). Anaplastic tumors often have complex karyotypes, suggesting they have a higher degree of genomic instability than their nonanaplastic counterparts (14, 16). It is therefore possible that genomic instability itself drives anaplastic change, and increased myc gene dosage is a secondary effect.

To further explore the hypothesis that c-Myc overexpression causes anaplastic changes in medulloblastoma, we developed a model system using medulloblastoma cell lines engineered to stably overexpress c-Myc. These lines showed increased rates of growth and apoptosis in vitro and in vivo and exhibited a markedly anaplastic histopathologic appearance in flank xenograft tumors. Analysis of a large set of pathologically diverse primary medulloblastomas also showed a significant association between elevated myc RNA levels comparable with those in our model system and anaplasia.

Materials and Methods

Constructs and cell culture. The plasmid pMYCEGFP was constructed by cloning the human c-myc cDNA (19) downstream of the cytomegalovirus (CMV) promoter in the EcoRI and SalI sites of pIRESEGFP (Clontech, Palo Alto, CA), pDHLuc (20) was used to assess myc activity; pTK-ren was purchased from Promega (Madison, WI). Qiagen (Valencia, CA) plasmid purification kits were used according to the manufacturer’s instructions in preparing DNA.

DAOY, D283, and D425 medulloblastoma (American Type Culture Collection, Manassas, VA) cell lines were grown in MEM Zn2+ option (Richter’s modification) medium supplemented with 10% fetal bovine serum (FBS). U228 medulloblastoma cells were grown in DMEM/F-12, 10% FBS and all lines were incubated at 37°C, 5% CO2. Medium and serum were purchased from Invitrogen (Carlsbad, CA).

Transfections were carried out using the FuGene reagent (Roche Diagnostics, Indianapolis, IN) at a ratio of 3:2 (FuGene/DNA, volume/mass)
according to the manufacturer's instructions. Cells were plated at a density of 20,000 to 40,000 per well in 24-well plates and collected 24 to 48 hours following transfection. The relative luciferase activities were measured using the Promega Dual Luciferase Reporter Assay kit. The standard luciferase activity was normalized using the Renilla luciferase activity.

Stable clones were derived from DAOY or UW228 cells transfected with either pMYCEGFP or pRISEEGFP. Following 48 hours of incubation, G418 was added to a concentration of 500 µg/mL. Cells were cultured in the selection medium for an additional 2 weeks. Clones were screened for green fluorescence by microscopy following single-cell plating in 96-well plates. To avoid unique insertion events, multiple clones from two different transfection experiments were derived and characterized. All myc+ lines have been continuously passaged for >18 months without any obvious change in phenotype.

**In vitro growth analysis.** Cells were plated at a density of 5,000 to 15,000 viable cells per well in 24-well plates on day 0. Cell number and viability were assessed using the Guava PCA and Viacount reagent according to instructions (Guava Technologies, Hayward, CA). The cells were collected by trypsin-EDTA treatment, pelleted by gentle centrifugation, and resuspended in Guava Viacount reagent for measurement on the Guava PCA. The Guava Viacount reagent contains two fluorescent dyes, one cell permeable and one impermeable, except in cells that have lost membrane integrity (propidium iodide [PI]). This allows assessment of the fraction of cells that are viable (membrane intact, no PI staining), dead (high PI staining), and intermediate or midapoptotic. Multiple replicate wells were assayed per time point in each experiment, and each subclone was examined in two to three independent experiments. Each time point represents the mean fold increase in the day 0 cell number from all scored wells; error bars display SE.

Cell cycle was also assessed on the Guava PCA following the manufacturer's technical report. Cells were plated in six-well plates at a density of 50,000 per well on day 0 and collected on day 3. After overnight fixation in 70% ethanol, the cells were pelleted, washed, incubated in PI staining solution (25 µg/mL PI in PBS, 0.1% Triton X-100) for 30 minutes, and analyzed on the Guava PCA. Each experiment represents a minimum of 5,000 gated events from each of three wells. Cell cycle fractions were assigned using Guava software.

**Soft agar assays.** Viable cells (n = 3,000) were plated in the appropriate medium in 0.5% agar over a 1% agar base and covered in medium with 10% serum. Medium was changed every 4 days for 10 to 21 days. For colony assessment, the plates were washed with PBS, stained with 0.5% Wright's stain in PBS, and counted with Chemidoc XRS (Becton Dickinson, San Diego, CA) at 10-sensitivity.

**Flank xenograft tumors.** Viable cells (4 × 106 DAOY-derived or 2 × 106 UW228-derived) were mixed 1:1 with Matrigel (BD Biosciences, San Jose, CA) and a total volume of 0.25 mL was injected s.c. into the flank of ether-anesthetized 5-week-old female nu/nu mice. Measurements of tumor size in long and short axes were made weekly and estimates of volume were made by the formula: width2 × length × 0.52 (21). At 8.5 to 9.5 days, the mice were sacrificed and tumors were excised. Following weighing, the tumors were split, half was snap frozen for subsequent protein and RNA analysis and half was fixed in formalin. To assess the apoptotic and proliferative indices, primary antibodies were used on formalin-fixed, paraffin-embedded xenograft tumor tissue. 3.3'-Diaminobenzidine immunoperoxidase staining was done on formalin-fixed, paraffin-embedded xenograft tumor tissue using the Vectastain Elite system according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA). Cleaved caspase-3 (rabbit monoclonal, Cell Signaling Technologies, Beverly, MA) was diluted 1:100 and MIB-1 (DAKO, Carpinteria, CA) was diluted 1:1,000; primary antibody incubations were for 45 minutes at room temperature. Positive cell counts were determined from randomly selected high-power fields in all tumors by a neuropathologist (T.W.A.) blinded to tumor genotype.

**RNA and protein preparation and analysis.** Total RNA from cultured cells or tumor xenografts was prepared using Qiagen RNasea kits according to instructions. A RNase-free DNase (Qiagen) step was added to reduce genomic DNA contamination. Primary medulloblastomas were obtained from the Department of Pathology, Johns Hopkins University School of Medicine, with institutional review board approval. Classification of histopathologic subtype was determined by one of two board-certified neuropathologists (C.G.E., or P.C.B.). RNA from medulloblastoma tumor samples snap-frozen at the time of surgery was prepared either with a Qiagen kit or using Trizol reagent (22). Whole-cell protein extracts were prepared as described previously (23).

Quantification of c-myc mRNA was done using a two-step real-time PCR method. Total RNA (500-2,000 ng) was reverse transcribed to cDNA using random hexamers and Moloney murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA). β-Actin and c-myc levels were determined using purchased Applied Biosystems PDARs and Taqman Master Mix on a Bio-Rad (Hercules, CA) iCycler (50°C × 2 minutes, 95°C × 10 minutes, 45 cycles of 95°C × 15 seconds, 60°C × 60 seconds) in 20 μL reaction volumes. A standard curve was generated using dilutions of DAOY cDNA and the unknown values were determined using the lycerase software. Each unknown (1-10 ng starting material) was run in triplicate or greater.

A SYBR Green–based real-time PCR assay was used to quantify levels of N-myrc RNA. Primers (forward 5'-TGAAGGAGGAGATGAGAGA-3' and reverse 5'-GTGACCGTCTGTTGAGGA-3') were designed using Primer3 express software to cross an intron/exon boundary and give a product of 80 bp. Template cDNA was added to 20 μL reactions containing 200 primer concentrations and 1× SYBR Green Master Mix (Applied Biosystems) and run on a Bio-Rad iCycler (95°C × 10 minutes, 45 cycles of 95°C × 15 seconds, 60°C × 60 seconds). Standard curves were generated using D283 cDNA serial dilutions. Relative expression values were normalized to β-actin levels, determined separately.

Whole-cell protein extracts (30-50 μg) were run on SDS-PAGE in Tris-glycine buffer, electrophoretically transferred to Nitran membranes, and blocked overnight at 4°C overnight in PBS with 5% NFDM. c-Myc protein levels were determined using 9E10 or N262 primary antibody hybridization (Santa Cruz Biotechnology, Santa Cruz, CA; 1:500 and 1:250 dilution, respectively) followed by anti-mouse or anti-rabbit secondary antibody (KPL, Inc., Gaithersburg, MD; 1:5,000 dilution) and visualization by chemiluminescence (Western Lightning, Perkin-Elmer, Foster City, CA). Densitometric quantitation was carried out using Chemidoc XRS software.

**Statistical analysis.** GraphPad Prism 4 statistical software (GraphPad Software, San Diego, CA) was used for statistical analysis. Error bars represent SE unless indicated otherwise.

**Results**

Characterization of medulloblastoma cells stably overexpressing c-myc. To understand the relationship between c-Myc activity and medulloblastoma growth characteristics, we selected two medulloblastoma cell lines, DAOY and UW228, which have not been described to have amplifications in the myc locus (24, 25). As outlined, the c-myc cDNA was cloned downstream of the CMV immediate-early promoter in pMYCEGFP. A series of stable transfectants carrying either this plasmid or the parent vector were derived via G418 selection and characterized. During the selection process, it was noted that pMYCEGFP transfection resulted in more EGFP-positive cells than pEGFP transfections, implying that there may be some selective advantage in c-Myc overexpression (data not shown). As shown in Fig. L4, c-myc mRNA levels as measured by a Taqman real-time PCR assay were induced 10- to 30-fold in the various DAOY-myc and UW228-myc lines (designated DAOY-1-2, 13-15, UW13-15) compared with either the parent or the vector-transfected lines (DAOYV1, UWV1). These increases in mRNA level are similar to those seen in the upper quartile of primary human tumors (Fig. 2) and approach levels of other cell lines (D425 and D283) known to have high c-Myc expression (data not shown), suggesting that our model falls into a biologically relevant range. Protein levels rose in parallel with mRNA expression (Fig. 1B). This protein was...
Overexpression of c-myc alters cell growth characteristics in vitro. As shown in Fig. 3A and B, the cell lines overexpressing c-myc had increased growth rates when cultured in standard conditions. The DAOY parent line basal growth rate was higher than that of UW228, with confluence achieved in 6 days versus 7 to 8 days, respectively, in these experiments. Myc overexpression resulted in higher numbers of cells throughout the logarithmic growth phase. Statistical analysis of grouped control DAOY versus DAOYM lines on day 5, just before growth plateau, revealed a 40% increase in fold change of cell number, with a mean of 9.1 for DAOY controls and 12.8 for DAOYM lines ($P = 0.002$, unpaired, two-tailed $t$ test). Similar analysis of control versus UWM lines on day 6 showed a 2-fold increase ($P < 0.001$), with a mean of 12.7 for UW controls and 25.2 for UWM lines. Although the data shown in Fig. 3A and B represent measurements of viable cells, the increase in growth rate was even more marked when comparing total cell

**Figure 1.** Levels and activity of c-myc are increased in a series of stably transfected DAOY and UW228 medulloblastoma cell lines. A. c-myc mRNA levels, determined by quantitative reverse transcription-PCR (RT-PCR), are increased 10- to 30-fold in c-myc–transfected cell lines (DAOYM2, DAOYM14, and UWM13-15) compared with parent (DAOY and UW228) or vector-transfected (DAOYV11 and UWV1) cells. Columns, averages of more than three independent determinations, normalized to $\beta$-actin levels; bars, SE. B. Western blots of protein extracts, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) included as a loading control, document similar increases in c-Myc protein levels. C. c-Myc transcriptional activity was measured using a transient transfection assay with the pLDHluc plasmid positively regulated by c-Myc. Results of two experiments run in triplicate. Bars, SE.

**Figure 2.** Increased myc mRNA levels in anaplastic medulloblastoma. Quantitative real-time RT-PCR was used to measure c-myc (A) or N-myc (B) transcript levels in primary human medulloblastoma, other primitive neuroectodermal tumors (PNET), and normal human cerebelli. The highest c-myc levels were observed in three tumors with diffuse anaplasia. Interestingly, three additional anaplastic medulloblastoma with low levels of c-myc mRNA (A, arrows) had high levels of N-myc mRNA (B, arrows). The three cerebelli with higher levels of N-myc were fetal samples. Bars, averages of values in each histopathologic group.

functional as evidenced by 1.5- to 2-fold increases in transcriptional activity measured by transient transfection of a c-Myc–sensitive promoter-reporter construct (Fig. 1C). The morphology of the transfected cells was not significantly altered in culture, although the UW-myc lines tended to have a more spindled appearance than their parent line. Both DAOYM and UWM lines had increases in the fraction of nonadherent cells and lower plating efficiencies when compared with the non-myc lines (data not shown) but have been passaged over 18 months without difficulty.
number (including viable, midapoptotic, and dead cells; data not shown). As the cells reached confluence, lines overexpressing c-myc had a significantly larger fraction of nonviable cells as seen in Fig. 3C. After 7 days in culture, well after the growth plateau, the DAOYM lines had a 2-fold larger percentage of PI staining dead cells than vector or parent lines, which was consistent with the subjective microscopic finding of increased numbers of floating cells. This phenomenon was similar in the UWM lines. In addition to its effects, increasing rates of apoptosis, c-Myc is a known regulator of cell cycle entry. However, the fraction of cells in S or G2-M phases was only slightly larger in the DAOYM lines than in controls (Fig. 3D). The finding that the parent line had a large proportion of cells in active cell cycle likely represents a feature of standard culture conditions, where growth factors and nutrient supply are not limiting. Increased c-myc activity may therefore only result in a small decrease in the quiescent cell population.

Overexpression of c-Myc greatly enhanced colony-forming ability in nonadherent conditions (Fig. 4). Both parent DAOY and UW228 cell lines inefficiently form colonies in soft agar. Experiments with DAOYM lines resulted in three to eight times more colonies compared with parent DAOY or the vector-transfected line DAOYV11. UW228M lines had a remarkably higher rate of colony formation than the parent line, with a 10- to 40-fold increase in number. The colonies formed by the myc-transfected DAOY lines were larger and generally more robust, a phenomenon that was also more pronounced in the UW228-derived cell lines (Fig. 4, inset). In both DAOYM and UW228M lines, the relative level of c-Myc in each subclone did not precisely correlate with their growth characteristics or clonogenicity. This suggests the possibility of a threshold level above which c-Myc does not have an additional additive effect on growth.

Increased c-Myc results in more rapid tumor growth in vivo. Given the limitations of in vitro analysis, we next examined the effect of c-Myc overexpression on growth of s.c. flank xenograft tumors in nu/nu mice by comparing the growth of six DAOYM-2 tumors to eight DAOY tumors. As shown in Fig. 5,
the DAOYM-2 line resulted in significantly larger tumors as measured by either weekly volumetric assessment (Fig. 5A) or final tumor weight (P < 0.05, unpaired, two-tailed t test; Fig. 5B and C) compared with those generated by the parent DAOY cell line. Other DAOYM lines also resulted in larger tumors and similar histopathologic changes (data not shown). High levels of c-myc RNA (Fig. 5D) and protein (Fig. 5E) were maintained in these tumors, confirming the stability of pMYCEGFP activity. As in the soft agar assays, overexpression of c-Myc had a much more profound effect on UW228 xenograft tumor formation. The parent line did not form tumors in any of six injections (tissue excised after 8.5 weeks showed only residual Matrigel and scar tissue). However, UWM14 or UWM15 cells resulted in tumors in 2 of 2 and 4 of 6 injections, respectively, as documented by histopathology (see Fig. 6E). These tumors were on average much smaller than those formed even by the parent DAOY line (Fig. 5C) but were clearly neoplasms on slide review, indicating that c-Myc overexpression in the UW228 cell line was required for efficient xenograft tumor formation.

As shown in Fig. 6A, c-myc markedly altered the histopathologic appearance of the DAOY xenograft tumors. Whereas the parent DAOY tumors consisted of a relatively homogeneous population of cells with bland nuclei and small nucleoli, the DAOYM-2 tumors had a more malignant appearance. Large areas of confluent apoptosis or necrosis very similar to the "apoptotic lakes" seen in primary LC/A medulloblastoma tumors were present in all sections examined. The nuclear morphology was notable for increased atypia and macronucleoli, again similar to primary tumors with the LC/A phenotype. Interestingly, scattered "cellular wrapping" was seen in the DAOYM-2 tumors (data not shown). Histologic analysis of H&E stains of the UWM tumors revealed remarkably similar features (Fig. 6F).

Unlike the in vitro analysis that only showed a mild increase in the cell fraction outside of G1-G0, DAOYM2 tumors had an ~2-fold increase (mean = 83) in the number of mitotic figures counted per 10 high-power fields compared with DAOY (mean = 48; Fig. 6B, with additional comparison with a primary LC/A tumor). Similarity, Ki-67 positivity was nearly doubled (Fig. 6C). Myc also increased apoptosis ~4-fold as measured by cleaved caspase-3 staining (Fig. 6D). All of these measurements between the two groups of tumors were different with a statistical significance of P < 0.05 (mitotic figures) or P < 0.01 (Ki-67 and cleaved caspase-3 staining) by unpaired, two-tailed t test. The lack of xenograft tumor formation by any UW228 control cell line precluded comparative analysis; however, the mitotic rate was robust in the UWM-derived tumors, averaging 25 mitotic figures per 10 high-power fields.

Primary medulloblastomas with diffuse anaplasia have high levels of c-myc or N-myc mRNA. Amplification of either the c-myc or the N-myc locus has been strongly associated with medulloblastoma anaplasia. We have reported previously that positive in situ staining for c-myc RNA correlated significantly with anaplasia (26). To further examine the association between myc and anaplasia in human tumors and to determine if c-myc levels in our model fell into the range observed in human tumors, we analyzed both c-myc and N-myc mRNA levels for 56 primary samples by real-time PCR: 8 medulloblastoma with diffuse anaplasia, 24 medulloblastoma with nonanaplastic (classic) histology or only focal anaplasia, 8 nodular-desmoplastic medulloblastoma, 6 primitive neuroectodermal tumors (PNETs including 2 medulloepithelioma), and 10 normal cerebellum. As shown in Fig. 2, high levels of c-myc mRNA were commonly found in the tumors with diffuse anaplasia (4 of 8 tumors, with values ranging 0.06-12.42) but infrequently in other nonanaplastic medulloblastomas and PNETs. Nodular medulloblastomas and normal cerebellum had very low levels of c-myc (with values ranging 0.17-1.13). This was a statistically significant finding both by one-way ANOVA (P = 0.0025) comparing all five groups and by unpaired t test (P = 0.0012) comparing diffusely anaplastic tumors with all other medulloblastoma samples. High levels of N-myc mRNA were found in many tumor samples and fetal cerebellum. There was no significant correlation with elevated N-myc and any one tumor subtype; however, several patterns did emerge. Medulloblastomas with a nodular phenotype, which is commonly associated with dysregulation of the Hedgehog signaling pathway, had high N-myc levels, consistent with previous reports of N-myc being a downstream target of Hedgehog signaling (27). Additionally, of the four medulloblastomas with diffuse anaplasia and low c-myc levels, three had markedly elevated N-myc (Fig. 2, arrows). Thus, very high levels of c-myc or N-myc are strongly associated with diffuse anaplastic features in medulloblastoma.

Discussion

There are several molecular markers under investigation for their prognostic significance in medulloblastoma. Molecular
Figure 6. Overexpression of c-Myc promotes anaplastic changes in flank xenograft tumors. A, H&E-stained sections of DAOY- and DAOYM2-derived tumors (left and middle) and a human LC/A medulloblastoma (right). Myc overexpression in DAOY cells results in increased nuclear size, prominent nucleoli, and regions of necrosis/confluent apoptosis, all features also seen in human LC/A medulloblastoma. Original magnification, ×40 (left to right) and ×600 (inset). B, as in human LC/A medulloblastoma (left), mitotic activity was brisk in xenografts with high c-Myc (middle). Original magnification, ×600. The number of mitotic figures per 10 high-power fields in DAOYM2 xenografts was significantly higher than in DAOY xenografts (P < 0.01, unpaired, two-tailed t test). C, the Ki-67 proliferation index was also significantly higher in DAOYM2 tumors (P < 0.001, unpaired, two-tailed t test). Original magnification, ×100. D, immunohistochemical stains for cleaved caspase-3 were used to highlight apoptotic cells in DAOY and DAOYM2 xenografts. Original magnification, ×100. Both single apoptotic cells and confluent regions of apoptosis were detected. The number of single apoptotic cells per 10 high-power fields was significantly higher in high c-Myc xenografts (P < 0.001, unpaired, two-tailed t test). E, photomicrographs of H&E-stained sections of UW228- and UWM14-derived tumors. Original magnification, ×40 (left and middle) and ×600 (right). The UW228 “tumor” is actually normal tissue with residual Matrigel. The low-power view highlights an area of necrosis/confluent apoptosis similar to that seen in the DAOYM tumors. The high-power view shows the similar nuclear features seen in primary LC/A medulloblastoma and DAOYM-derived tumors.
features, such as isolated chromosome 17p loss, HIC1 gene hypermethylation, and elevated ERBB2 protein expression, have been associated with poor outcomes, whereas high TRKC expression has been associated with improved survival [briefly reviewed by Fisher et al. (28)]. Immunopositivity for p53 has also been shown to be a negative prognostic factor (6, 29, 30). In addition, there is ample evidence that myc plays an important role in medulloblastoma biology. Increased c-myc activity in medulloblastoma cell lines has been shown to occur through both gene amplification (31) and altered transcriptional regulation (32). In primary tumor specimens, two large retrospective analyses showed that high levels of c-myc mRNA were found in a much larger proportion of cases than could be explained solely by gene amplification and were associated with poor outcome (33, 34). These studies did not evaluate anaplasia, so a direct link between expression of c-myc and histologic subtype could not be made. However, we have shown recently that elevation in c-myc mRNA level as identified by in situ hybridization is associated not only with poor clinical outcome but also with anaplasia (26).

Here, we show that there is a causative link between increased c-Myc and anaplasia in medulloblastoma. We created a series of stably transfected medulloblastoma cell lines expressing high levels of c-Myc. The higher levels of c-myc mRNA level were mirrored by increases in protein and activity, implying that mRNA measurement was a reasonable surrogate for relative activity. In vitro, only subtle changes in cellular morphology were noted, but increased rates of growth and apoptosis occurred. The ability to form colonies in soft agar was greatly increased in terms of both quantity and colony size. Myc overexpression also resulted in significantly larger flank xenograft tumors and, most remarkably, a microscopic phenotype akin to the LC/A subtype of medulloblastoma. Similarities to human LC/A medulloblastoma included increases in nuclear atypia, apoptosis, and mitotic index along with macronucleoli and occasional "cellular wrapping." Myc overexpression was required for efficient establishment of flank xenograft tumors in the UW228 cell line. Moreover, increased Myc activity in UW228 cells resulted in highly similar LC/A changes, indicating that the effects of Myc on phenotype are not confined to the DAOY cell line. Finally, analysis of a large set of primary medulloblastoma tumors showed strong correlation between c-myc mRNA level and diffuse anaplasia. Taken together, these data strongly suggest that increased c-myc activity not only is associated with anaplasia in medulloblastoma but also induces anaplasia.

The role of N-myc is potentially very interesting and one that we have addressed before (26). It may be that it fulfills a similar role as c-Myc in the development of anaplasia, as there does seem to be some functional redundancy between the two proteins. N-myc can restore viability in c-myc−/− mice (35) and a modified ornithine decarboxylase promoter can detect both c-myc and N-myc activity (36). However, N-myc, unlike c-myc, has been identified as a crucial downstream target of the sonic hedgehog (shh)/PTCH pathway both in the normal developing cerebellum and in nodular medulloblastomas (27, 37–40). This is consistent with our finding of higher levels of N-myc mRNA in fetal cerebella and nodular tumors. c-myc mRNA, conversely, is not found in high levels in either of these tissues. This dichotomy, the prognostic value of c-myc expression in nonanaplastic tumors, and the role of N-myc in anaplastic progression will require further elucidation.

The distinctive changes in the histopathologic appearance and in vivo aggressiveness induced by c-Myc are much more profound than the effects in vitro. Some of the differences are a matter of degree; there are increases in growth and apoptosis in culture that are more pronounced when analyzed in vivo. However, the ability of Myc to increase growth in nonadherent conditions implies that there are changes that cannot be quantified by standard cell culture alone. In our xenograft model, additional factors, such as angiogenesis and/or decreased dependence on limiting growth factors, may also play a role. This seems highly probable, given that the cell intrinsic changes leading to an increased growth rate in
of some medulloblastomas, its increased expression and activity leads to transformation (42). In other cases, such as breast cancer, c-myc dysregulation leads to more aggressive disease but is insufficient to cause tumorigenesis alone [reviewed by Jamerson et al. (43)]. The ability of Myc alone to induce medulloblastomas may be limited as well. In a mouse model system, Rao et al. found that combining c-Myc overexpression with ectopic shh production using their RCAS-TVA system resulted in more tumors than shh alone (44). In their hands, however, c-Myc by itself did not cause tumors, resulting only in areas of subependymal cellular proliferation. In contrast, the Li-Fraumeni, Gorlin, and Turcot tumor predisposition syndromes as well as mouse medulloblastoma models indicate that Myc regulates (extensively reviewed in ref. 41), this is not surprising.

We believe that, although c-myc may play a role in the initiation of some medulloblastomas, its increased expression and activity is primarily observed as tumors progress and become more aggressive. This can occur either at recurrence or in anaplastic subclones present in the specimen resected at initial presentation. As outlined in Fig. 7, we propose a multistep progression model, with c-myc oncogenes playing a critical role in the development of the LC/A phenotype in medulloblastoma (12). Genes or pathways associated in other studies with anaplasia, including TP53/ARF, ERBB2, OTX2, and hTERT, may also play a role in medulloblastoma progression (3, 47–49) How these specific molecular events relate to anaplastic progression has yet to be determined, but they may substitute in some way for c-myc dysregulation. Regardless, it is clear that c-Myc overexpression is sufficient to induce anaplasia in at least two medulloblastoma cell lines and is strongly associated with the anaplastic phenotype in primary tumors.

Acknowledgments

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